

Protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic ALS mice

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Received April 30, 2001; Revised and Accepted June 20, 2001

Amyotrophic lateral sclerosis (ALS) is mainly a sporadic neurodegenerative disorder characterized by loss of cortical and spinal motoneurons. Some familial ALS cases (FALS) have been linked to dominant mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1). Transgenic mice overexpressing a mutated form of human SOD1 with a Gly⁹³Ala substitution develop progressive muscle wasting and paralysis as a result of spinal motoneuron loss and die at 5 to 6 months. We investigated the effects of neurotrophic factor gene delivery in this FALS model. Intramuscular injection of an adenoviral vector encoding cardiotrophin-1 (CT-1) in SOD1^{Gly93Ala} newborn mice resulted in systemic delivery of CT-1, supplying motoneurons with a continuous source of trophic factor. CT-1 delayed the onset of motor impairment as assessed in the rotarod test. Axonal degeneration was slowed and skeletal muscle atrophy was largely reduced by CT-1 treatment. By monitoring the amplitude of the evoked motor response, we showed that the time-course of motor impairment was significantly decreased by CT-1 treatment. Thus, adenovirus-mediated gene transfer of neurotrophic factors might delay neurogenic muscular atrophy and progressive neuromuscular deficiency in ALS patients.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a common neurodegenerative disease in humans, involving motoneuron loss in the cortex, brainstem and spinal cord. It typically affects adults in their fifth decade, leading to progressive muscle wasting, paralysis and death within 3 to 5 years. ALS is mainly sporadic; however, 10% of cases are familial (FALS) and have

similar clinical and histopathological features. About 20% of FALS cases are associated with dominantly inherited mutations in the Cu/Zn superoxide dismutase gene (SOD1) (1). SOD1 cytosolic enzyme catalyzes the dismutation of superoxide radical into hydrogen peroxide and molecular oxygen, suggesting that reduced SOD activity could increase oxidative stress. However, transgenic mice expressing FALS-linked mutations in the SOD1 gene develop severe denervating and paralytic processes that resemble ALS without loss of SOD1 activity (2–5). These observations suggest that mutant SOD1 proteins acquire a novel gain of function that might contribute to the pathogenesis. Several hypotheses have been proposed to explain this gain of function. These include increased peroxidase activity, nitration of tyrosines via formation of peroxynitrite, metal toxicity, SOD1-aggregation-mediated toxicity or inhibition of glial glutamate uptake (for reviews see 6,7). However, to date, molecular mechanisms leading to selective motoneuron degeneration remain poorly understood.

Mice expressing the Gly⁹³Ala (G93A) mutation in exon 4 of the human SOD1 gene exhibit an autosomal dominant adult onset of motoneuron disease (2). The prominent and selective loss of spinal motoneurons leads to progressive paralysis, muscle wasting, atrophy, and death at 5–6 months. These mice provide a very useful animal model of FALS and have been used for identifying therapeutic agents. Antioxidants slow down the progression of the disease but have no effect on survival (8,9), whereas inhibitors of the glutamatergic system and copper chelators delay disease onset slightly and extend survival to a small extent (8,10). A creatine diet improves motor performance and slightly protects motoneurons from oxidative damage (11). Finally, promising results were obtained through overexpression of the Bcl-2 anti-apoptotic gene or caspase inhibitors; both approaches have been shown to delay motoneuron death (12–14), suggesting that apoptosis plays an important role in ALS pathogenesis.

In the absence of a clear understanding of the pathogenic process, neurotrophic factors have been hypothesized to be able to slow down motoneuron cell death and axonal degeneration

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and also to promote muscle reinnervation (for reviews see 15,16). Beneficial effects of neurotrophic factor overexpression have been reported in spontaneous models of motoneuron disease, *wobbler* and *pmn* mice (17–20). In *pmn* mice, we recently described the impressive neuroprotective effect of cardiotrophin-1 (CT-1) (21). This cytokine of the IL-6 family has been described as a very potent neurotrophic factor for spinal motoneurons in long-term culture and protects neonatal sciatic motoneurons from axotomy-induced cell death in rats (22). Its overexpression in *pmn* mice significantly reduces degeneration of facial motoneurons and phrenic axons and preserves the terminal innervation of skeletal muscles that is grossly disturbed in untreated *pmn* mice (21). We have already shown that intramuscular adenoviral gene transfer in neonatal mice results in the efficient and long-term expression and delivery of a secreted protein (23).

Using this gene therapy approach, we have now evaluated the neuroprotective potential of CT-1 in *SOD1^{G93A}* mice. Neonatal transgenic mice received a single injection of adenoviral vector, and both functional and histopathological parameters were evaluated to assess the effects of CT-1 gene delivery on disease progression.

RESULTS

Effects of CT-1 gene delivery on general behavior of *SOD1^{G93A}* mice

Neonatal transgenic *SOD1^{G93A}* mice were injected with a total dose of 10^8 plaque forming units (PFU) of AdCT-1 or AdLacZ into five muscles (both gastrocnemii, both triceps brachii and long dorsal muscles of the trunk). We have already shown that a similar injection protocol results in long-term gene expression (20,21). In this study, gene expression was assessed from day 130–210 after gene transfer in injected muscles and also in serum. As shown in Figure 1A, adenoviral CT-1 transcripts were detected by RT-PCR in all AdCT-1 injected muscles until very late stages. In sera, CT-1 bioactivities were measured using a ciliary ganglion neuron survival assay. At 130 days after gene transfer, the sera of all analyzed *SOD1^{G93A}*-treated mice contained elevated CT-1 bioactivities, in contrast to sera from control *SOD1^{G93A}* mice (Fig. 1B). We thus conclude that CT-1 gene expression persisted over the course of the study.

To assess the effects of treatment on general behavior, *SOD1^{G93A}* mice were regularly weighed (Fig. 2A). With disease progression, untreated *SOD1^{G93A}* mice had reduced weight gain and even lost weight at end-stage, as described previously. Compared with untreated animals, AdCT-1-treated mice had a slightly reduced weight gain on the first weeks after adenoviral injection, which probably reflects the side effects of CT-1 as observed in AdCT-1-treated *pmn* mice (21). At 120 days of age however, the weight of AdCT-1-treated *SOD1^{G93A}* mice reached values close to normal and remained higher than the weight of untreated *SOD1^{G93A}* mice even in end-stage disease.

CT-1 delays disease onset and modestly improves survival of *SOD1^{G93A}* mice

The appearance of the first signs of motoneuron disease in *SOD1^{G93A}* mice was assessed by rotarod analysis. Mice were tested on a rotarod on a weekly basis from day 60–190 of life.

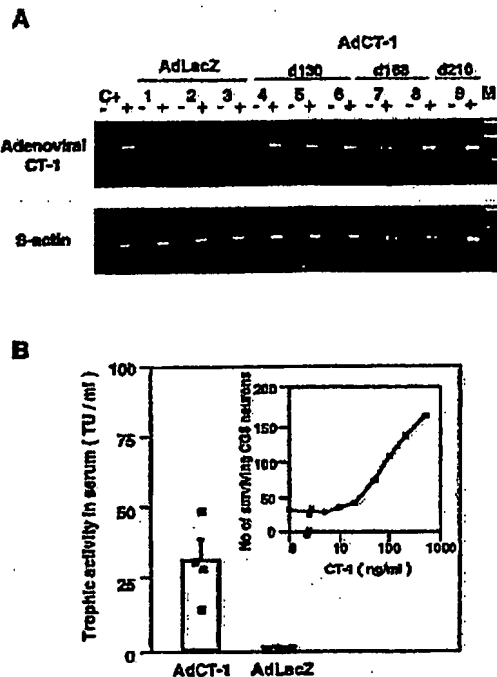


Figure 1. CT-1 expression in *SOD1^{G93A}* mice after intramuscular gene delivery. (A) Adenoviral CT-1 transcripts were detected by RT-PCR in gastrocnemius muscles of AdCT-1-injected mice aged 130 days (lanes 4–6), 168 days (lanes 7–8) and 210 days (lane 9). In contrast, no signal was detected in control AdLacZ-injected mice (lanes 1–3). C+, RNA from AdCT-1-infected 293 cells. Control RT-PCR reactions were performed using primers specific for β -actin. Reactions with (+) and without (–) reverse transcriptase. (B) CT-1 bioactivities in mouse sera as analyzed by a ciliary ganglion neuron survival assay. Elevated CT-1 bioactivities [in tropic units (TU) per milliliter] were detected in the sera of AdCT-1-injected *SOD1^{G93A}* mice aged 130 days ($n = 4$) but not in control mice ($n = 4$). One tropic unit is defined as the serum dilution allowing half-maximal survival of neurons. The number of surviving neurons as a function of the concentration of recombinant CT-1 protein is shown in the inset.

The onset of motor deficit was defined as the first day a mouse could not remain for 3 min on the rotarod turning at a speed of 15 r.p.m. The average age of motor deficit onset in AdCT-1-treated *SOD1^{G93A}* mice was 153 ± 7.8 days (mean \pm SEM, $n = 7$) compared with 126 ± 8.5 days ($n = 6$) in untreated *SOD1^{G93A}* mice (Fig. 2B). CT-1 treatment thus delayed motor deficit onset by 27 days (Fisher's test, $P = 0.039$).

As shown in Figure 2C, the CT-1 treatment prolonged the mean survival of *SOD* mice by about 13 days (mean \pm SEM: untreated, 172.6 ± 4.6 days, $n = 25$; CT-1-treated, 185.9 ± 3.9 days, $n = 46$, $P = 0.034$). When compared with a small number of AdLacZ-injected *SOD1^{G93A}* mice, the mean survival of CT-1 treated *SOD1^{G93A}* mice also tended to improve (LacZ, 176.3 ± 5 days, $n = 12$) albeit this difference did not reach statistical significance ($P = 0.094$; Fig. 2C).

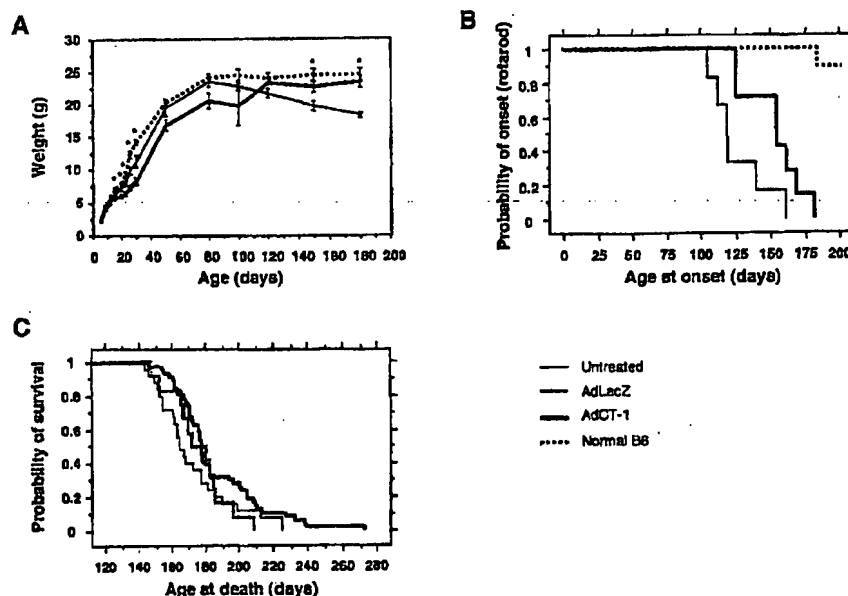


Figure 2. General behavior of *SOD1*^{G93A} mice. (A) Body weight variations in AdCT-1-treated mice and control groups. Asterisks denote the statistical difference between AdCT-1-treated mice compared with untreated animals. Average mean number of mice analyzed: wild-type, $n = 17$; AdCT-1, $n = 14$; untreated, $n = 8$. (B) Cumulative probability of onset of severe motor deficits. Motor function was assessed with the rotarod at 15 r.p.m. from day 60–190. Testing was terminated arbitrarily at 3 min. The onset of motor deficiency was defined as the day the mouse could not complete the test. CT-1 delayed onset of motor deficits by 27 days compared with untreated *SOD1*^{G93A} mice (Fisher's test, $P = 0.039$). (C) Cumulative probability of survival in *SOD1*^{G93A} mice. CT-1 improved the survival of *SOD1*^{G93A} ($n = 46$) by about 11–13 days as compared with untreated ($n = 25$) or AdLacZ-injected ($n = 12$) mice (AdCT-1 versus untreated, $P = 0.034$; AdCT-1 versus AdLacZ, $P = 0.094$).

CT-1 improves neuromuscular function of *SOD1*^{G93A} mice

We used electromyography (EMG) to characterize more precisely the progressive impairment in neuromuscular function in *SOD1*^{G93A}-treated mice. Changes in EMG parameters are detected far earlier than onset of tremor or motoneuron loss (24,25). In this study we recorded compound muscle action potential (CMAP) amplitudes in the gastrocnemius muscle from 80 to 180 days after injection to assess the extent of impairment of motor units after CT-1 treatment. Up to 110 days, CMAP values in gastrocnemius muscle did not differ in either CT-1-treated or control *SOD1*^{G93A} mice (Fig. 3A). Thereafter, CMAP amplitude decreased at a significantly lower rate in AdCT-1-treated compared with untreated *SOD1*^{G93A} mice (-0.44 mV/day in *SOD1*^{G93A}-untreated mice; -0.29 mV/day in CT-1-treated animals, regression analysis and Fisher's test, $P < 0.0001$). In end-stage disease (180 days), the CMAP amplitude remained significantly higher in CT-1-treated mice than in untreated *SOD1*^{G93A} mice.

Distal motor latencies were measured in parallel with CMAP in gastrocnemius muscle (Fig. 3B). Motor latencies were increased in all *SOD1*^{G93A} mice compared with normal mice. Up to 140 days of age, no significant difference was observed between untreated and AdCT-1-treated *SOD1*^{G93A} mice. At later time points however, distal motor latencies were significantly

lower in CT-1-treated *SOD1*^{G93A} mice compared with untreated *SOD1*^{G93A} mice (Fisher's test, $P = 0.0003$).

Histological effects of CT-1

Histological analysis of *SOD* mice was performed at 130 days of age. The medial and lateral gastrocnemius muscles were dissected and weighed (Fig. 4). The weight of AdLacZ-treated muscles of *SOD1*^{G93A} mice was only 46% of the weight of wild-type muscles (mean \pm SEM: AdLacZ, 72.2 ± 3.5 mg, $n = 5$; wild type, 156.4 ± 8.4 mg, $n = 7$). In sharp contrast, gastrocnemius muscles from AdCT-1-treated mice weighed nearly twice as much (131.7 ± 8.6 mg, $n = 10$) than those of AdLacZ-injected mice ($P < 0.001$), and only 16% less than those of wild-type mice ($P = 0.04$). These data suggest that CT-1 treatment slowed muscle atrophy.

Next, cross-sections of gastrocnemius muscles were stained for myofibrillar ATPase (Fig. 5). In untreated mice, neurogenic muscle atrophy was observed with the presence of angulated small atrophic fibers surrounded by hypertrophic fibers. Both medial and lateral parts of the gastrocnemius muscle were affected. The presence of a few muscle fibers with central nuclei indicated a certain extent of muscle regeneration. However, we did not observe changes in either the proportion of fiber type or fiber type grouping (data not shown). CT-1 treatment markedly reduced denervation atrophy. Only rare

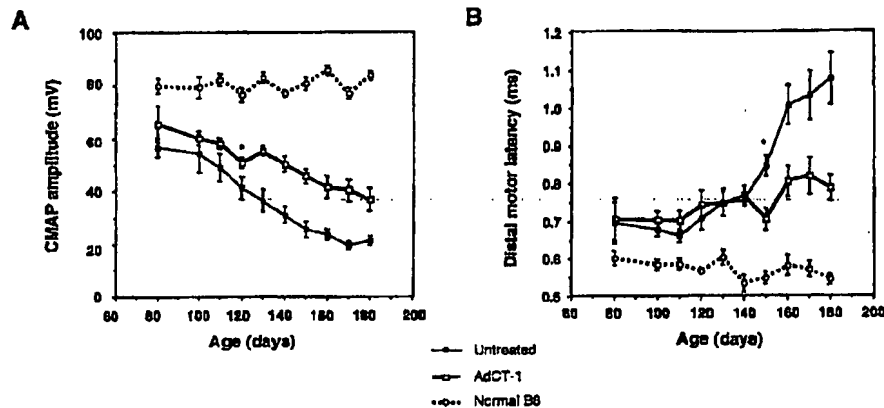


Figure 3. Time course of evoked motor response impairment in *SOD1^{G93A}* mice. (A) Evoked CMAP amplitude and (B) distal motor latency were recorded in the gastrocnemius muscle after stimulation of the sciatic nerve in AdCT-1-treated (mean number of analyzed mice, $n = 9$), untreated *SOD1^{G93A}* mice ($n = 8$) and normal littermates ($n = 7$). Measurements were made at 80 days and every 10 days from 100 to 180 days. Mean data from left and right muscles are presented (\pm SEM). Asterisks denote the first day on which data are significantly different between AdCT-1-treated mice and untreated *SOD1^{G93A}* mice ($P < 0.05$).

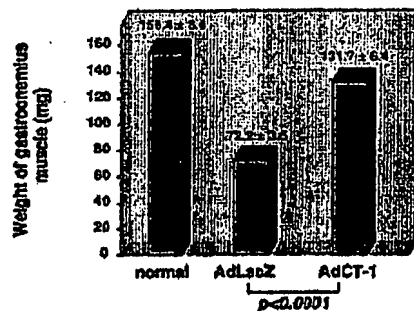


Figure 4. Weight of gastrocnemius muscles of *SOD1^{G93A}* mice aged 130 days. Note that CT-1 gene delivery significantly delayed muscle atrophy in *SOD1^{G93A}* mice as compared with control AdLacZ-injected *SOD1^{G93A}* mice ($n = 5$). AdCT-1-treated muscles ($n = 10$) were lighter than corresponding wild-type muscles ($n = 7$). Mean \pm SEM.

angulated atrophic fibers were observed in the medial and lateral gastrocnemius of AdCT-1-treated mice. We next measured the fiber diameter of about 150–200 fibers per mouse in the lateral part of the gastrocnemius. A decrease in the mean fiber diameter was apparent in untreated *SOD* mice (mean \pm SEM: $20.2 \pm 0.8 \mu\text{m}$, $n = 3$) as compared with normal mice ($40.6 \pm 0.6 \mu\text{m}$, $n = 3$). The size distribution in untreated mice demonstrated significant atrophy with a high number of very small fibers (38.2% $< 12 \mu\text{m}$) and the presence of some hypertrophic fibers with a diameter superior to normal value (4.7% $> 60 \mu\text{m}$). After CT-1 treatment ($n = 3$), the fiber size distribution was much more uniform and close to normal, although the mean diameter was reduced ($21.5 \pm 0.3 \mu\text{m}$).

In order to further confirm that CT-1 treatment reduced muscle denervation, muscle cross sections were treated for

non-specific esterase staining and positive fibers were counted. Strong esterase activity was observed in $38.3 \pm 4.8\%$ muscle fibers from untreated *SOD1^{G93A}* mice (total number of fibers, $n = 320$), whereas only $0.6 \pm 0.3\%$ fibers ($n = 1008$) were stained in AdCT-1-treated *SOD1^{G93A}* mice and no esterase-positive fibers were observed in normal mice (Fig. 5).

Axonal degeneration in *SOD1^{G93A}* mice has been demonstrated in the ventral lumbar roots and in the phrenic nerve at end-stage disease (2,26). Here, we counted myelinated axons in phrenic nerves of mice aged 130 days (Fig. 6). Phrenic nerves of AdCT-1-treated *SOD1^{G93A}* mice contained 30% more myelinated fibers than control *SOD1^{G93A}* mice (AdCT-1, 200 ± 6 , $n = 10$; AdLacZ, 153 ± 7 , $n = 10$, $P < 0.001$).

DISCUSSION

Skeletal muscles transduced with an adenoviral vector were used to deliver CT-1 to motoneuron terminals, supplying them with a continuous source of biologically synthesized neurotrophic factor. We have already shown that such gene therapy approach was effective in slowing down motoneuron cell body and axonal degeneration in the *pnn* mice (21). Untreated, these mice suffer from progressive motor neuropathy with prominent axonal degeneration and muscle atrophy. Lower motoneuron cell death is observed in end-stage disease mainly in the facial nucleus and to a lesser extent in the lumbar spinal cord (17,20,27). However, the mechanism by which *pnn* motoneurons undergo cell death is unknown since the *pnn* mutation remains undiscovered. Here, mice transgenic for a mutated form of the human *SOD1* gene (*SOD1^{G93A}*) associated with FALS provided an ideal model to test our experimental therapeutic approach for ALS. Loss of lower motoneurons in the spinal cord leads to hindlimb tremor, muscle wasting and progressive muscle atrophy resulting in death at 5 to 6 months of age. Direct intramuscular injection of the AdCT-1 vector results in efficient and stable expression of

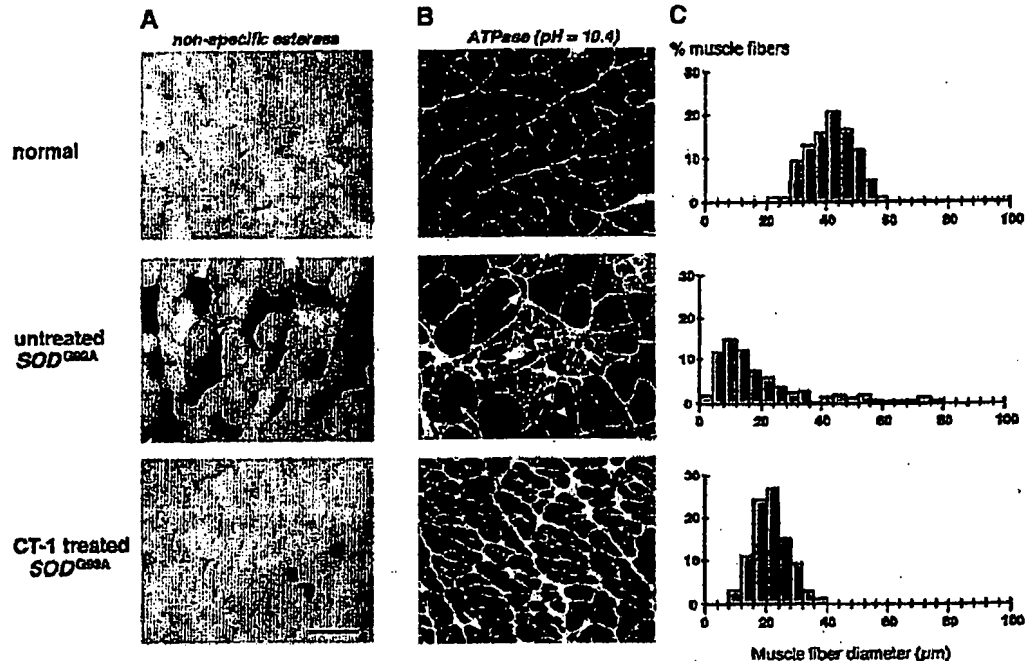


Figure 5. Histological analysis of denervation-induced muscle atrophy in untreated and CT-1-treated *SOD^{G93A}* mice. Cross-sections of gastrocnemius muscles of mice aged 130 days were stained for non-specific esterase (A) and alkaline ATPase (B) histochemistry. (A) Numerous denervated fibers as indicated by strong esterase activity were present in untreated *SOD^{G93A}* mice ($n = 3$). In contrast, stained fibers were rare after CT-1 treatment ($n = 3$). (B) In untreated *SOD^{G93A}* mice, large groups of angulated and atrophic muscle fibers (thick arrows) were observed after ATPase staining. Most often, hypertrophic fibers (thin arrow) were present in atrophic regions. Signs of regeneration were also evident with fibers showing central nuclei (arrowheads). CT-1 treatment markedly attenuated denervation atrophy. No gross muscle fiber abnormalities were observed in AdCT-1-treated *SOD* mice, although fiber area was smaller than in normal mice ($n = 3$). (C) Morphometric analysis confirmed changes in the distribution of muscle fiber diameter. Megahistograms were constructed by combining three histograms from the lateral gastrocnemius of each group. Scale bar, 100 μm.

CT-1 in muscles for at least 210 days, resulting in CT-1 activity in bloodstream. General behavior of AdCT-1-treated mice, as measured by body weight, was improved in end-stage disease. Furthermore, CT-1 delayed the onset of severe motor deficits as evaluated by improved performance in the rotarod test. The decline of EMG parameters was also significantly delayed. Both CMAP amplitude and distal motor latency were improved by AdCT-1 injection. We conclude that CT-1 overexpression is able to slow down degradation of the neuromuscular function in FALS transgenic mice.

At the age of 130 days, the weight of gastrocnemius muscle in untreated *SOD^{G93A}* mice was half that in normal mice in accordance with previous observations (28). In contrast, muscle atrophy was slowed dramatically after CT-1 treatment, although some cytokine cachexic effects have probably resulted in loss of muscle fiber area (29,30). Histological observations confirmed that neurogenic atrophy was clearly reduced by CT-1 treatment. Several hypotheses might explain this effect. CT-1 expression may have stimulated sprouting which might increase innervation of muscles and protect them

against atrophy. However, we did not observe any changes in the fiber typing pattern in gastrocnemius muscles. Furthermore, our observation of terminal innervation in intercostal and abdominal muscles, either at the 130th day or at the terminal stage of the disease (data not shown), did not support this hypothesis since sprouting or branching of terminal axons did not seem to be increased compared to untreated mice. Another hypothesis is that CT-1 may have protected muscles from denervation by slowing down motoneuron and axonal degeneration. A denervation marker, strong esterase activity (31), was evident in untreated *SOD* mice, whereas it was nearly absent in AdCT-1-treated mice. We also observed an increased number of myelinated axons in phrenic nerves of CT-1-treated mice compared with control AdLacZ-injected *SOD^{G93A}* mice, providing evidence that axonal degeneration was clearly reduced by CT-1 treatment. These results are consistent with our previous observations in *pnn* mice, where the terminal innervation pattern appeared to be preserved by CT-1 gene delivery though no marked reinnervation was noted (21). Furthermore, raised CMAP amplitudes in AdCT-1-treated mice indicate

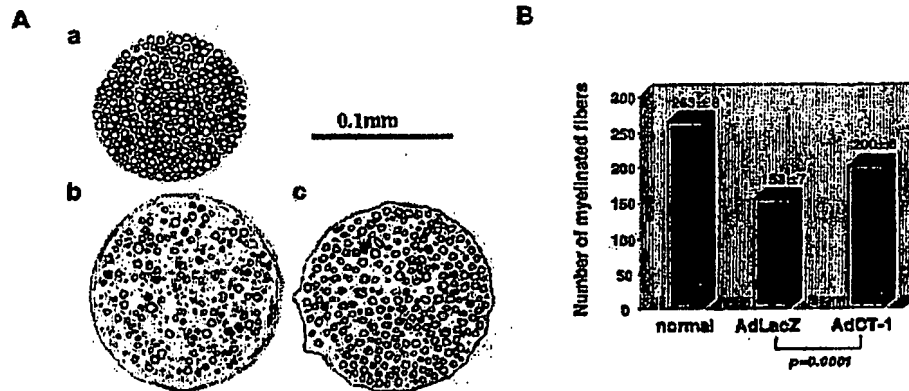


Figure 6. Phrenic nerve axonal counts in 130-day-old *SOD1*^{G93A} mice. (A) Histological analysis of phrenic nerves from a normal C57Bl/6 mice (a), AdLacZ-injected *SOD1*^{G93A} mice (b) and AdCT-1-treated mice aged 130 days (c). (B) Intramuscular CT-1 gene delivery to *SOD1*^{G93A} mice ($n = 10$) resulted in a significantly greater number of surviving myelinated axons compared with control AdLacZ-injected *SOD1*^{G93A} mice ($n = 10$). Compared with wild-type littermates ($n = 4$), CT-1 treatment reduced axonal degeneration in FALS mice by 40%. Mean \pm SEM.

that more muscle fibers were innervated (24). Altogether, these results suggest that CT-1 delays degeneration distally at the nerve terminals and neuromuscular junctions, and thereby prevents the gradual decline in the size of the motor units. Finally, myotrophic effects of CT-1 may have contributed to reduce muscle atrophy. Such myotrophic effects of CT-1 have been well described in cardiac muscle (32–34), but have not yet been reported in skeletal muscle. However, they have been described for the related cytokines CNTF and LIF after nerve section (30,35) and, for CNTF, also in *wobbler* mice (36). It is also known that the LIFR β and gp130 subunit receptors of the CT-1 receptor are expressed by skeletal muscle and that their expression is increased in denervated muscle (35).

Recently, a myoblast-based GDNF gene therapy was applied in *SOD1*^{G93A} mice (37). Myoblasts infected with GDNF retroviral vectors, and thus secreting this factor, were grafted into hindlimb muscles of *SOD1*^{G93A} mice. This prevented loss of large diameter spinal motoneurons and delayed the onset of the disease. However, no effect on survival was reported. In our study, the mean lifespan of *SOD1*^{G93A} was increased after AdCT-1 injection although the observed gain of two weeks was relatively small. This result contrasts with the dramatic protective effects of CT-1 on axonal degeneration and muscle atrophy. Several hypotheses might explain this discrepancy. First, survival pathways initiated by CT-1 might not be efficient enough to circumvent the apoptotic events activated very early in the *SOD1*^{G93A} disease (14). Second, the increased circulating CT-1 levels we measured in late stage animals might not have been available at sufficient amounts to degenerating motoneurons. Indeed, axonal transport, which is impaired in various ALS transgenic mice (38,39), might have lead to insufficient supply of the trophic factor to cell bodies thereby limiting its action. Finally, there is increasing evidence that subpopulations of motoneurons differ in their vulnerability to degeneration (40) and also in their response to trophic factors (41). In cell culture or during normal development for instance, CT-1 seems to be required only for the survival of

subpopulations of motoneurons (42). In *SOD1*^{G93A} mice, some fast-type neuromuscular synapses are already lost around day 50, whereas slow-type synapses resist until late phases of disease (40). Our results indicate that CT-1 treatment did not significantly improve the earliest electromyographic abnormalities of *SOD1*^{G93A} mice but had pronounced effects on disease progression. It can thus be speculated that CT-1 protected selected subpopulations of degenerating motoneurons or neuromuscular synapses.

In conclusion, we demonstrate that CT-1 gene delivery has neuroprotective effects in a transgenic mouse model of FALS. The results previously observed in the *pmn* model were confirmed in these mice. The marked slowing of axonal degeneration and muscle denervation leads to a protection against the loss of neuromuscular function and a delayed onset of severe motor dysfunction. These results suggest that a similar therapy resulting in continuous neurotrophic factor production by transduced skeletal muscles could also improve the course of ALS in humans. Associated perhaps with other drugs that protect motoneuron cell bodies from apoptosis, CT-1 gene therapy could be one of the promising innovative treatments that are anxiously awaited in ALS, today a largely untreatable disease with an appalling prognosis.

MATERIALS AND METHODS

Animals

Male transgenic mice with the G93A human *SOD1* mutation (G1 line) were provided by Transgenic Alliance (Saint-Germain-sur-L'Arbresle, France). The gene copy number was confirmed by Southern blot to be about 13–19 as previously shown (2). Male transgenic mice were mated with background-matched B6SJL/F₁ wild-type females (Iffa Credo, L'Arbresle, France). The progeny were genotyped by the polymerase chain reaction (PCR) amplification of toe DNA from 3-day-old animals. PCR primers used were: exon 3, 5'-TTCTGTTCCTTCTCACTGT-3'

and 5'-TCCCCTTTGGCACTTGTATT-3'; exon 5, 5'-TGTTGGGAGGAGGTAGTGATTA-3' and 5'-AGCAGAGTGTGTTAGTTTATG-3'. Expected product sizes for exons 3 and 5 were 500 and 760 bp, respectively. Internal PCR control was achieved by amplifying part of the mouse globin gene with primers, 5'-GATCATGACCGCCGTAGG-3' and 5'-CATGA-CTTGTCCCAGGCTT-3' in the same reaction. Mice were killed at various time points for *CT-1* gene expression analysis and histological measurements. All animal experiments were carried out in accordance with institutional guidelines for care and use of laboratory animals.

Intramuscular injection of adenoviral vectors

Construction of AdCT-1 and AdLacZ has been described previously (21,43). Briefly, the AdCT-1 vector drives the expression of murine CT-1 cDNA fitted with the β NGF signal peptide under the control of the RSV LTR promoter. Viruses were amplified in 293 cells and purified on CsCl gradients according to standard methods (44). Titers were 3×10^{11} PFU/ml for AdCT-1 and 1.8×10^{11} PFU/ml for AdLacZ. Neonate mice (5–6 days) were briefly anesthetized by hypothermia. Vectors were diluted to 1×10^8 PFU/100 μ l of PBS and injected into three muscle groups, the gastrocnemius (25 μ l each), the triceps brachii (15 μ l each) and the long muscles of the dorsal trunk (20 μ l).

CT-1 gene expression

Transgene expression was determined over the course of the disease. The gastrocnemius muscles were dissected and homogenized in RNA-B reagent (Bioprobe, Montreuil, France) for total RNA purification. RT-PCR analysis of adenoviral *CT-1* transcripts was performed with the specific primers described previously (21). Control RT-PCR reactions were carried out using β -actin primers. CT-1 bioactivity in sera was determined at 130 days of age using a survival assay of ciliary ganglion neurons essentially as detailed previously (45). Briefly, ciliary ganglion neurons from 8-day-old chicken embryos were isolated, seeded in 96-well culture dishes and cultured in chemically defined medium to which sera or recombinant CT-1 protein (R&D, Abingdon, UK) were added at various dilutions. Neuronal survival in triplicate wells was assessed 48 h after plating by adding the vital dye MTT and counting the blue cells having metabolized the MTT.

Onset of motor dysfunction

A rotarod (Leticia LB8200, Italy) was used to evaluate motor function. Mice were placed on the rotating rod at 15 r.p.m. The time each mouse remained on the rod was recorded. If the mouse remained on the rod for 3 min, the test was stopped and scored as 3 min. The test was performed twice and only the maximum time was taken into account. Assays were done on a weekly basis from day 60–190. The onset of motor deficits was defined as the first day a mouse could not remain on the rotarod for 3 min.

Electrophysiological recordings

Evoked CMAP amplitudes were evaluated with a RACIA (M.E.I., Montreuil, France) EMG apparatus as described previously (24). Mice were deeply anesthetized with 60 μ g

sodium pentobarbital. The sciatic nerve was stimulated by single 0.2 ms supramaximal pulses through a concentric needle electrode (Dantec, 9013R0312, diameter 0.3 mm), and CMAPs were recorded from the medial part of the gastrocnemius with the same type of electrode. The peak-to-peak amplitudes and distal motor latencies of the evoked responses were measured three times in left and right muscles and averaged. Measurements were repeated every 10 days from 80 to 180 days of age in normal and *SOD1*^{G93A} mice.

Histological examination

To evaluate nerve and muscle degeneration, mice were killed at 130 days of age and during end-stage disease. Nerves were prepared as described previously (20). Briefly, mice were deeply anesthetized and phrenic nerves fixed *in situ* with 2.5% glutaraldehyde, 20 mg/ml sodium cacodylate before dissection. Nerves were post-fixed with osmic tetroxide and embedded in epoxy resin. 3 μ m thick cross-sections, taken at a similar level from the diaphragm, were stained with p-phenylenediamine. Myelinated fibers were counted under light microscopy.

Gastrocnemius muscles were dissected, weighed and frozen immediately in cooled isopentane and stored at -80°C . Serial 10 μ m thick sections were stained for myofibrillar ATPase under acid (pH 4.35; pH 5.53) and basic (pH 10.4) conditions using standard methods to allow identification of muscle fiber types I and II (46). ATPase stained sections were imaged using an image analysis system (Samba 2005 TITN, Alcatel, France). The size of muscle fibers was assessed by measuring the 'smallest fiber diameter'. Typically, 150–200 fibers were analyzed for each muscle. Some cross-sections were also stained for non-specific esterases using the α naphthyl acetate method. Briefly, sections were fixed in citrate-acetone-formaldehyde solution and incubated in cholinesterase inhibitor (eserine 10 μ M) for 30 min at 4°C . After washing, sections were incubated in staining medium (0.1 M Trizma maleate, 2 mM sodium nitrite, 0.3 mg/ml Fast Blue BB base, 0.25 mg/ml α -naphthyl acetate) for 30 min at 37°C protected from light. After intensive washes, slides were counterstained in hematoxylin solution and mounted in aqueous medium.

Statistical analysis

Statistical analysis was performed using repeated measures analysis of variance (ANOVA). Differences between individual groups were evaluated using the Fisher post-hoc test.

ACKNOWLEDGEMENTS

We thank Dr Philippe Kennel for advice on electromyographical recordings, Dr Vincent Mallet for his help in statistical analysis and Mariane Bjaerg for expert preparation of the phrenic nerve samples. Thanks are due to Huguette Collin and Andrée Rouché (INSERM U523, Institut de Myologie, Paris, France) for their help in muscle histology and histometry. We are grateful to Dr Jean-Paul Concordet for insightful discussions and Dr Toni L. Williamsom for critical reading of the manuscript. This work was supported by the Association Française contre les Myopathies (AFM), Fondation pour la Recherche Médicale and INSERM. T.B. and J.-C.L. are the recipients of an AFM fellowship.

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